Remarks/Arguments:

Claims 2-3, 7-8, 28, 37, 45, 47, 59, 61, 81-82, and 85-92 are canceled without prejudice. Claims 1, 6, 26-27, 35-36, 44, 46, 58, and 60 are amended. More specifically, claims 1, 6, 26, 35, 44, and 58 are amended by incorporating the limitations from claims 81, 82, 86, 88, 90, and 92, respectively. Claim 27 is amended by incorporating the limitations from claims 26, 28, and 85-86. Claim 36 is amended by incorporating the limitations from claims 35, 37, and 87-88. Claim 46 is amended by incorporating the limitations from claims 44, 47, and 89-90. Claim 60 is amended by incorporating the limitations from claims 58, 61, and 91-92. Claims 35 and 58 are also amended to correct minor typographical errors. No new matter is introduced.

Claims 1, 6, 12-13, 26-27, 35-36, 44, 46, 52-53, 58, 60, and 74 are pending in the application. Reexamination and reconsideration of the application, as amended, are respectfully requested.

CLAIM OBJECTIONS

Claims 35-37, 58-61, 87-88, and 91-92 are objected to because claims 35 and 58 recite "interferon, alpha-2b." Applicants have deleted the extra comma in this term.

Claim 58 is further objected to because it recites "a round melanoma biochemotherapy." Applicants have replaced the term with "a round of melanoma biochemotherapy."

In light of the foregoing, Applicants respectfully submit that the objections are overcome and should be withdrawn.

CLAIM REJECTIONS UNDER 35 USC § 112, SECOND PARAGRAPH

Claims 27-28, 36-37, 46-47, 60-61, and 87-92 are rejected as being indefinite for reciting inconsistent terms in independent claims and their dependent claims.

Applicants have amended claims 27, 36, 46, and 60 such that they no longer depend from claims 26, 35, 44, and 58, respectively. Claims 28, 37, 47, 61, and 87-92 have been canceled without prejudice.

In light of the foregoing, Applicants respectfully submit that the rejections are overcome and should be withdrawn.

CLAIM REJECTION UNDER 35 USC § 102(b)

Claim 26 is rejected as being anticipated by Soengas et al. (2001) Nature 409:207-211 ("Soengas"). Applicants respectfully traverse.

Claim 26 is directed to a method of monitoring melanoma progression. The method comprises providing a melanoma tissue sample or a blood sample containing DNA from a human subject suffering from melanoma and analyzing DNA markers comprising D12S1657, D12S393, D12S1706, and D12S346 on the DNA. The loss of heterozygosity of any of D12S1657, D12S393, D12S1706, and D12S346 indicates the progression of melanoma in the subject.

As presented in Applicants' previous responses to Office Actions, Soengas compares the expression of APAF-1 in metastatic and primary melanoma samples (page 207, right column, line 14 – page 208, left column, line 2). However, Soengas fails to show that the expression of APAF-1 necessarily correlates with LOH of D12S1657, D12S393, D12S1706, or D12S346 in metastatic and primary melanoma (see, e.g., Soengas, page 207, right column, Figures 1b and 1c, samples 6, 11, and 16; Figure 3 of the present specification, patients 30-32).

Further, Soengas speculated without any evidence that loss of APAF-1 may be associated with disease progression (page 207, right column, line 20 – page 208, left column, line 4). However, as the Examiner pointed out in the Advisory Action

dated April 30, 2009, claim 26 involves LOH of D12S1657, D12S393, D12S1706, or D12S346, not loss of APAF-1. In addition, in Soengas, the APAF-1 gene was mapped to a position between D12S1657 and D12S393. As pointed out at page 28, line 27 – page 29, line 6 of the present specification, the correct location of the APAF-1 gene is between D12S1706 and D12S346. Consequently, if one skilled in the art relies on the value of LOH of APAF-1 as determined in Soengas to predict the progression of melanoma, the prediction would not be correct.

Therefore, Soengas cannot anticipate claim 26, because it fails to teach the association of LOH of D12S1657, D12S393, D12S1706, or D12S346 with melanoma progression. Applicants thus respectfully request that the rejection be withdrawn.

CLAIM REJECTIONS UNDER 35 USC § 103

(1) Claims 1-3, 6-8, 12-13, 74, and 81-82 are rejected as being unpatentable over Soengas in view of U.S. Patent No. 6,156,504 to Gocke et al. ("Gocke"). Applicants respectfully traverse.

Among the rejected claims, claims 1 and 6 are independent claims. Claims 1 and 6 involve detecting or analyzing one or more DNA markers selected from the group consisting of D12S1657, D12S393, D12S1706, and D12S346 on DNA existing as acellular DNA in a human subject. As presented in Applicants' previous responses to Office Actions, Soengas analyzes D12S1657, D12S393, D12S1706, and D12S346 on cellular DNA from melanoma samples. Gocke discloses the detection of extracellular tumor-associated nucleic acid in blood plasma or serum in general, but does not suggest at all the detection of D12S1657, D12S393, D12S1706, or D12S346 on acellular DNA. Since neither Soengas nor Gocke indicates the presence of D12S1657, D12S393, D12S1706, or D12S346 on acellular DNA, the two references, either alone or in combination, do not even add up to the claimed invention. Further, one skilled in the art would not have reasonably expected that D12S1657,

D12S393, D12S1706, or D12S346, or LOH of D12S1657, D12S393, D12S1706, or D12S346, could be detected on acellular DNA, because the presence of microsatellite markers or LOH of such markers is not necessarily identical or consistent in tumor cells and as acellular DNA. See, e.g., Fujiwara et al. (1999) Cancer Research 59:1567-1571 (a copy of which is attached hereto as Exhibit A), page 1569, right column, Table 2, patients 1, 3, 6, 8-10, 14-17, 19-20, 23, and 30-31.

Therefore, claims 1 and 6 (as well as claims 12-13 and 74 dependent from claim 1 or 6) are non-obvious over Soengas and Gocke, because the two references, either alone or in combination, fail to teach each and every limitation of the claims and provide no reasonable expectation of success. Claims 2-3, 7-8, and 81-82 have been canceled without prejudice. Withdrawal of the rejections is thus respectfully requested.

(2) Claims 35 and 58-59 are rejected as being unpatentable over Soengas in view of O'Day et al. (1999) Journal of Clinical Oncology 17:2752-2761 ("O'Day"). Applicants respectfully traverse.

Claim 35 is directed to a method of predicting the efficacy of a melanoma biochemotherapy. The method comprises providing a melanoma tissue sample or a blood sample containing DNA from a human subject suffering from stage IV melanoma prior to administration of a biochemotherapy and analyzing DNA markers comprising D12S1657, D12S393, D12S1706, and D12S346 on the DNA. The biochemotherapy comprises dacarbazine, cisplatin, vinblastin, interferon alpha-2b, IL-2, and tamoxifen. The loss of heterozygosity of any of D12S1657, D12S393, D12S1706, and D12S346 indicates poor efficacy of the biochemotherapy in the subject.

Claim 58 is directed to a method of determining the probability of responsiveness to a round of melanoma biochemotherapy. The method comprises providing a melanoma tissue sample or a blood sample containing DNA from a

human subject suffering from stage IV melanoma prior to administration of biochemotherapy and analyzing DNA markers comprising D12S1657, D12S393, D12S1706, and D12S346 on the DNA. The biochemotherapy comprises dacarbazine, cisplatin, vinblastin, interferon alpha-2b, IL-2, and tamoxifen. The loss of heterozygosity of any of D12S1657, D12S393, D12S1706, and D12S346 indicates a low probability of responsiveness to the biochemotherapy in the subject.

As presented in Applicants' previous responses to Office Actions, claims 35 and 58 relate to the association of LOH of D12S1657, D12S393, D12S1706, or D12S346 with poor efficacy of a melanoma biochemotherapy or a low probability of responsiveness to the biochemotherapy in a human subject. In contrast, Soengas discloses that APAF-1 negative melanoma cell lines are resistant to ADR, a chemotherapeutic agent (page 209, left column, lines 8-10).

APAF-1 negative cells are cells expressing little APAF-1 (Soengas, page 208, left column, line 10). As discussed above, the expression of APAF-1 does not necessarily correlate with LOH of D12S1657, D12S393, D12S1706, or D12S346. Further, it is well known in the art that in vitro experiments do not necessarily reflect the conditions in vivo. See, e.g., Walter and Miller's textbook of radiotherapy: radiation physics, therapy, and oncology, by C. K. Bomford, I. H. Kunkler, Joseph Walter, and H. Miller, edition: 6, illustrated, published by Elsevier Health Sciences, 2003, page 611, right column, lines 11-20 (a copy of which is attached hereto as Exhibit B). As such, Soengas cannot render obvious claim 35 or 58, because it fails to teach the association of LOH of D12S1657, D12S393, D12S1706, or D12S346 with poor efficacy of a melanoma biochemotherapy or a low probability of responsiveness to the biochemotherapy in a human subject.

O'Day cannot cure the defect of Soengas, and was not relied on by the Examiner for such. Instead, O'Day was relied on by the Examiner for teaching a

biochemotherapy comprising dacarbazine, cisplatin, vinblastin, interferon alpha-2b, IL-2, and tamoxifen.

Therefore, claims 35 and 58 are non-obvious over the cited art at least for lack of reasonable expectation of success. Claim 59 has been canceled without prejudice. Withdrawal of the rejections is thus respectfully requested.

(3) Claims 44-45 are rejected as being unpatentable over Soengas in view of Taback et al. (2001) Cancer Research 61:5723-5726 ("Taback"). Applicants respectfully traverse.

Claim 44 is directed to a method of determining the probability of survival. The method comprises providing a melanoma tissue sample or a blood sample containing DNA from a human subject suffering from a stage III or IV melanoma and analyzing DNA markers comprising D12S1657, D12S393, D12S1706, and D12S346 on the DNA. The loss of heterozygosity of any of D12S1657, D12S393, D12S1706, and D12S346 indicates that the subject has a low probability of surviving melanoma.

Soengas discloses the inactivation of APAF-1 in metastatic melanoma (page 207, left column, Abstract). It suggests nothing about the association of LOH of D12S1657, D12S393, D12S1706, or D12S346 with a low probability of surviving melanoma. As such, Soengas cannot render claim 44 obvious. In this connection, Applicants point out that, as discussed about, Soengas does not suggest the association of LOH of D12S1657, D12S393, D12S1706, or D12S346 with melanoma progression or resistance to melanoma biochemotherapy in a human subject.

Taback cannot cure the defect of Soengas, and was not relied on by the Examiner for such. Instead, Taback was relied on by the Examiner for teaching the association of LOH of some microsatellite markers in stages III and IV melanoma with a decreased probability of survival. However, the microsatellite markers tested in Taback are not D12S1657, D12S393, D12S1706, or D12S346.

Therefore, claim 44 is non-obvious over Soengas and Taback, because the two references, either alone or in combination, fail to teach each and every limitation of the claim and provide no reasonable expectation of success. Claim 45 has been canceled without prejudice. Withdrawal of the rejections is thus respectfully requested.

(4) Claims 52-53 are rejected as being unpatentable over Soengas in view of Taback, and further in view of Yu et al. (1999) Cancer 86:612-627 ("Yu"). Applicants respectfully traverse.

Claims 52-53 depend from claim 44. As mentioned above, Soengas and Taback, either alone or in combination, cannot render claim 44 obvious. Yu cannot cure the defects of Soengas and Yu, and was not relied on by the Examiner for such. Instead, Taback was relied on by the Examiner for teaching RLM and ITM.

Therefore, claim 44 is non-obvious over Soengas, Taback, and Yu, because the cited references, either alone or in combination, fail to teach each and every limitation of the claim and provide no reasonable expectation of success. So are claims 52-53 dependent from claim 44. Withdrawal of the rejections is thus respectfully requested.

RESPONSE TO ADVISORY ACTION AND NOTICE OF NON-COMPLIANT AMENDMENT DATED MARCH 13, 2009

(1) New issues and non-compliance

The term "melanoma" was inadvertently deleted from claim 44 and missing in the amendment to claim 46 in Applicants' 2/20/09 reply to the final Office Action dated November 20, 2008. Applicants have now added the term to claims 44 and 46. Applicants thus respectfully submit that the new issues and non-compliance have been overcome and should be withdrawn.

(2) Claim objections

Applicants intended to delete the extra comma between "interferon" and "alpha-2b" in claims 35 and 58 in the 2/20/09 reply to the final Office Action dated November 20, 2008. However, a stroke-through comma was inadvertently included in the amendment to claim 36. The deletion of the extra comma in claims 35 and 58 is now denoted by double brackets as suggested by the Examiner. Applicants have also deleted the stroke-through comma in claim 36. Applicants thus respectfully submit that the objections have been overcome and should be withdrawn.

(3) Claim rejections

With regard to Applicants' election of a combination of D12S1657, D12S393, D12S1706, and D12S346 in response to the Restriction Requirement, Applicants respectfully point out that, while the combination was elected for analysis, it was not elected for requiring all four markers to lose heterozygosity. The Examiner concurred with this interpretation in the Advisory Action dated April 30, 2009. Applicants thus respectfully submit that the rejections are improper and should be withdrawn.

CONCLUSION

In view of the foregoing, it is respectfully submitted that the application is in condition for allowance. Reexamination and reconsideration of the application, as amended, are requested.

If for any reason the Examiner finds the application other than in condition for allowance, the Examiner is requested to call the undersigned at the Los Angeles, California telephone number (310) 785-4600 to discuss the steps necessary for placing the application in condition for allowance.

Respectfully submitted,

HOGAN & HARTSON L.L.P.

Date: May 20, 2009

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Exhibit A

[CANCER RESEARCH 59, 1567–1571, April 1, 1999]

Plasma DNA Microsatellites as Tumor-specific Markers and Indicators of Tumor Progression in Melanoma Patients[1]

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ABSTRACT

Multiple DNA microsatellites with frequent loss of heterozygosity (LOH) in melanomas have been demonstrated. The finding that free DNA is enriched in blood of melanoma patients prompted studies to determine whether tumor-specific DNA, such as DNA microsatellites exhibiting LOH, can be detected in blood and have clinical use. In this study, 57 advanced and 19 early clinically staged melanoma patients were assessed using 10 microsatellite markers on six chromosomes. Matched plasma and melanoma tissues from 40 patients showed significant concordance of LOH ($P < 0.0001$). The frequency of LOH microsatellite markers detected in plasma significantly increased in more advanced-staged patients. At locus *D3S1293*, LOH detection showed significant correlation to clinical disease progression ($P = 0.02$). Additionally, the combination of LOH microsatellite markers D9S157 and D3S1293 ($P = 0.01$), D9S157 and D1S228 ($P = 0.05$), and D11S925 and D3S1293 ($P = 0.01$) were significantly correlated to progression of different clinical stages of disease. These studies indicate that tumor-specific LOH markers in plasma have a potential clinical use as diagnostic and prognostic markers in melanoma patients.

INTRODUCTION

Recent advances in tumor genetics have revealed that genesis and progression of tumors follow an accumulation of multiple genetic alterations, including inactivation of tumor suppressor genes and/or activation of proto-oncogenes (1). Frequent LOH[3] of DNA microsatellites on specific chromosomal regions have been reported in various types of malignancies. The frequency of LOH in tumors, along with homozygous deletions at specific chromosome sites in tumor cells, suggests the involvement of putative tumor suppressor genes or oncoproteins related to tumorigenesis and/or tumor progression. LOH analysis, combined with genetic linkage analysis on pedigrees of familial cancer (2–6) or homozygous deletion analysis (7–9), has identified candidate tumor suppressor genes. Allelic losses or microsatellite alterations on specific chromosomes are the most common genetic alterations observed in a wide variety of malignancies (10–15). Recently, LOH analysis of tumor tissues has been shown to be of prognostic value (10, 14, 16). To date, however, microsatellite analysis for LOH has been primarily carried out using labor intensive assessment of archival paraffin tumor sections.

There is evidence that naked DNA is released, enriched, and remains stable in the blood of cancer patients (17–18). Recently, tumor-specific DNA has been detected in the plasma and serum of lung, head and neck, and colon cancer patients (19–23). This suggests that cell-free plasma/serum is a source for detecting cancer-specific DNA markers. In the past, tumor-associated markers such as proteins/glycoproteins have been used for diagnosis or prognosis of progression in patients. However, the specificity of these assays is limited because the majority of these markers are not tumor-specific and are found in normal cells. To date, tumor-specific genetic markers have been assessed primarily in tumor biopsies. However, in advanced-staged patients, surgery is not always performed, which limits the availability of tumor tissue for genetic assessment. The detection of tumor-specific genetic markers in cancer patients at distant sites from the tumor, such as in the blood, provides a unique and valuable tumor genetic marker assay for diagnosis and prognosis.

Melanoma can be a highly aggressive cancer that becomes very difficult to manage clinically when disease progression occurs. Patients diagnosed with early-stage primary melanoma (AJCC stages I and II) who undergo surgical treatment have a low incidence of disease recurrence and usually a positive prognosis (24–26). However, when recurrence of disease occurs, it is often difficult to manage. There is considerable variability in the extent and type of disease progression in AJCC stage III and stage IV patients (25, 26). Therefore, the genetic analysis of recurrence and stages of disease spread may aid in improving prediction of disease progression and in determining the most effective strategy for treatment. The stepwise biological progression of melanomas from benign nevi to malignancy has been documented (27). However, the corresponding genetic analysis of individual stages of tumor progression is limited. The detection and understanding of genetic changes relating to melanoma progression is not well understood, particularly during disease progression from AJCC stage II to stage IV.

In melanoma, deletions and mutations of several known tumor suppressor genes, such as *TP53* and *CDKN2*, have been reported; however, they occur at a low frequency (28–30). The tumor suppressor gene *CDKN2* at 9p21 encoding $p16^{ink4}$ protein is associated with sporadic and familial melanoma (29–31). There are frequent homozygous deletions and LOH at the microsatellite loci *9p21* region in melanoma. However, the absence or mutation of CDKN2 has not been found frequently or well correlated with tumor progression. Additionally, there are other potential promising DNA markers (such as microsatellites) with frequent LOH on chromosome loci *1p36*, *3p25*, *6q22-q26*, *10q24-q26*, and *11q23* that have been reported in melanomas (13, 16, 32–35).

In this study, we examined the plasma of 57 advanced and 19 early clinically-staged melanoma patients using a panel of 10 microsatellite markers representing six chromosomal regions. Of these patients, 40 with matched tumor lesions available were assessed. The study demonstrated that multiple LOH markers can be detected in the plasma of melanoma patients and not in healthy donors. The study also demonstrated that melanomas release tumor-specific genetic markers in blood that highly correlate to the patients' respective melanoma lesion. There was a significant correlation between frequency and combinations of specific microsatellite markers with LOH to clinical disease progression.

Received 10/19/98; accepted 1/28/99.

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[1] Supported in part by National Institutes of Health Grant NCI PO CA13917 Project II.

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[3] The abbreviations used are: LOH, loss of heterozygosity; AJCC, American Joint Commission on Cancer; RT-PCR, reverse transcription-PCR.

MATERIALS AND METHODS

Specimens. Blood (5 ml) was collected in sodium citrate-containing tubes (Becton Dickinson, Franklin Lakes, NJ) from a total of 76 patients diagnosed with melanoma at the John Wayne Cancer Clinic. Similarly, blood was drawn from 20 healthy donor volunteers. The plasma was immediately separated from blood cells by differential centrifugation at 1000 × *g* for 15 min, filtered through a 13-mm serum filter (Fisher Scientific, Pittsburgh, PA) to remove any potential cells, and then cryopreserved at -30°C until DNA extraction. Blood was spotted on FTA blood stain cards (Fitzco, Minneapolis, MN) for normal genomic DNA extraction, as well as long-term storage. Respective WBCs from individual patients were used as normal DNA controls. Cells were separated by differential centrifugation from RBCs using Puregene RBC lysis solution (Gentra Systems, Minneapolis, MN). The cell pellet was then washed with PBS. Corresponding tumor tissues were microdissected from two to three 10-μm serial sections of formalin-fixed paraffin-embedded blocks, as previously described (36). All microdissected tissue sections were identified histopathology positive for malignant melanoma cells (28).

DNA Isolation. Control lymphocyte DNA was isolated using DNAzol (Molecular Research Center Inc., Cincinnati, OH). In brief, cell pellets were homogenized with 1 ml of DNAzol and precipitated by the addition of 0.5 ml of 100% ethanol. After centrifugation, precipitated DNA was then washed twice with 95% ethanol and resuspended in 10 mM Tris (pH 8)-1 mM EDTA buffer. Plasma (1 ml) was diluted at 60% with a solution of 0.9 M NaCl, 1% SDS, and proteinase K. The diluted plasma was shaken and incubated at 37°C overnight in the presence of an equal volume of phenol-chloroform isoamyl alcohol (25:24:1). After centrifugation for 15 min at 1000 × *g*, the aqueous phase was collected, extracted with an equal volume of phenol-chloroform isoamyl alcohol, and precipitated by isopropanol. Tumor lesions microdissected from 40 paraffin-embedded tissue blocks were incubated with xylenes at 37°C overnight (12). The pellet was recovered after centrifugation and washed twice with 1 ml of 100% ethanol. The remaining material was dried by vacuum centrifugation, incubated with proteinase K in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, and 0.5% Tween 20) at 37°C overnight and then boiled at 95°C for 10 min in a heat block.

Microsatellite Markers and PCR. Ten primer sets for PCR amplification of microsatellite markers were chosen on six chromosome arms. The following $CA_{(n)}$-repeat microsatellite markers were used: D1S214 at 1p36.3; D1S228 at 1p36; D3S1293 at 3p-3p24.2; D6S264 at 6q25.2–6q27; IGFIIR at 6q25-q27; D9S157 at 9p23-p22; D9S161 at 9p21; D10S212 at 10q26.12–10q26.13; D10S216 at 10q24-q26; and D11S925 at 11q23.3–11q24. Primer sets for PCR were obtained from Research Genetics, Inc. (Huntsville, AL), and sense primers were labeled with a fluorescent FAM or Cy5 dye. Genomic DNA (~50 ng) was amplified by PCR in 25-μl reactions containing 1 × PCR buffer (6.7 mM Tris, 16.6 mM ammonium sulfate, 6.7 mM EDTA, and 10 mM β-mercaptoethanol), 6 pmol of each primer, 1 unit of Taq DNA polymerase, 0.8 mM of each dNTP, and 1.5 mM $MgCl_2$. Forty PCR cycles were performed, with each cycle consisting of 30 s at 94°C, 30 s at 50–56°C, and 90 s at 72°C, followed by a final extension step of 72°C for 5 min.

LOH Analysis. PCR product (5 μl) mixed with 2 μl of loading dye (100% formamide, 2 mM EDTA, and 2% dextrane blue) was incubated at 95°C for 15 min. The concentrated samples were electrophoresed on a 6% denaturing PAGE containing 7.7 M urea at 1600V for 2 h. The fluorescent-labeled PCR product images were scanned by a fluorescent/optical Genomyx SC scanner (Genomyx Corp., Foster City, CA). After the image acquisition was completed, image files were analyzed by Adobe Photoshop software (Adobe, San Jose, CA). Densitometry analysis was performed with the imaging software ClaritySC (Media Cybernetics, Silver Spring, MD). Intensity calculations and intensity comparison of the specific alleles in lymphocytes, plasma, and tumor DNA were performed to evaluate for LOH. Tumor and plasma were scored as exhibiting LOH if there was an absence or more than a 50% reduction in the intensity of one allele compared with the respective allele in the normal matched lymphocytes.

Statistics. Correlation between patients' matched tumor and plasma samples for individual microsatellite markers were assessed using the Kappa agreement test. Logistic regression was used to test the association between the number of positive markers and AJCC Stage (37). Spearman correlation was estimated to evaluate the association between the number of positive markers, Breslow's thickness, and Clark's level. The χ^2 test was also used when data were cross-classified into a contingency table.

RESULTS

LOH Analysis in Plasma and Melanoma Biopsies. LOH was initially assessed at 10 different loci on six different chromosomes in paired tumor biopsies and plasma from 40 melanoma patients. DNA was extracted and detected from the plasma, tumor biopsies, and lymphocytes of all patients. The frequency of LOH varied between 23% and 53% in tumor biopsies and 4% and 33% in plasma for individual microsatellite markers in informative cases. For informative melanoma tumors (Table 1; Fig. 1), the most frequent microsatellite markers with LOH were detected at loci *D6S264*, *D9S161*, *D10S216*, and *D11S925*, respectively. Additionally, in informative plasmas from melanoma patients (Table 1; Fig. 1), the most frequent microsatellite markers with LOH were detected at loci *D10S216*, *D3S1293*, *D6S264*, and *D1S214*, respectively. Microsatellite markers *D6S264* and *D10S216* were among those loci that frequently had LOH for both plasma and tumor. The least frequent microsatellite marker for LOH detection was IGFIIR, in both plasma and tumor. Representative LOH in tumor and plasma as compared with lymphocyte DNA is shown in Fig. 2. LOH was not observed for any of the microsatellite markers tested on the lymphocytes and plasma DNA from 20 healthy donors.

A summary of LOH in tumor and plasma from 40 melanoma patients using a panel of 10 microsatellite markers is shown in Table 2. LOH for at least one microsatellite marker was shown in tumor samples of 34 of 40 (85%) patients and in plasma samples of 23 of 40 (58%) patients. Overall, a significant correlation ($P < 0.0001$) between LOH matched-paired plasma and tumor specimens of each individual microsatellite marker was shown. Twenty-one of 23 (91%) patients with LOH markers in plasma showed LOH in their respective tumors, when assessing all microsatellite markers. Two of these patients had LOH markers in plasma and not tumor. In 13 patients, LOH was observed in tumor and not plasma. In one interesting case, the plasma DNA showed LOH at *D3S1293* but not at *D9S157*, whereas the tumor sample of this patient showed LOH at both loci. These differences may be due to different metastases at various sites or clonal heterogeneity within the tumor, in which there are tumor cell colonies with or without specific microsatellite marker(s) LOH.

Plasma LOH Correlation with Different Clinical Stages of Melanoma. Plasma was assessed from 76 melanoma patients with different clinical stages (AJCC) of disease: 7 stage I; 12 stage II; 30 stage III; and 27 stage IV. The mean age of the patients was 51.6 ± 17.0 SD, consisting of 28 females and 48 males. The mean Breslow's thickness was 2.07 mm. The majority of the patients had a Clark's level of III or IV. In informative patient cases, a correlation between the frequency of plasma LOH and AJCC stage was performed (Table 3). The frequency of LOH was higher in more advanced stages of

Table 1 *Microsatellite analysis of paired tumor and plasma DNA in melanoma patients*

Microsatellite locus	Chromosome location	LOH in tumor/ informative cases	LOH in plasma/ informative cases
D1S214	1p36.3	9/24 (38%)	5/25 (20%)
D1S228	1p36	8/24 (33%)	5/26 (19%)
D3S1293	3p25	6/17 (35%)	6/21 (29%)
D6S264	6q25.2-q27	8/15 (53%)	4/15 (27%)
IGFIIR	6q26-q27	5/22 (23%)	1/25 (4%)
D9S157	9p23-p22	11/28 (39%)	3/30 (10%)
D9S161	9p21	11/25 (44%)	5/26 (19%)
D10S212	10q26.12-.13	6/15 (40%)	1/11 (9%)
D10S216	10q24-q26	5/12 (42%)	4/12 (33%)
D11S925	11q23.3-24	9/22 (41%)	2/24 (8%)

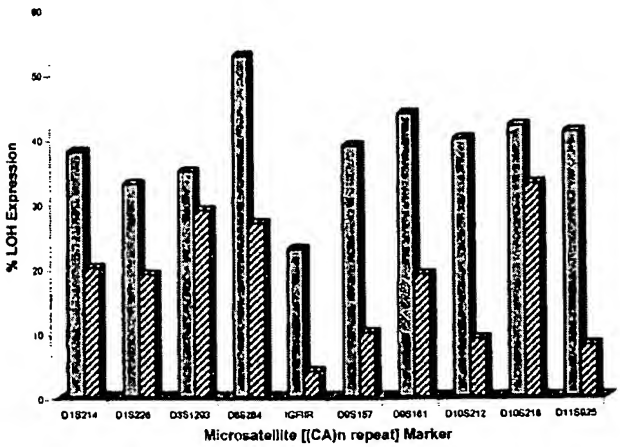

Fig. 1. Representative frequency of LOH in plasma and tumor of advanced melanoma patients at 10 microsatellite loci. Markers examined are indicated on the bottom of the figure. ■, frequency of LOH in tumor; ▨, frequency of LOH in plasma.

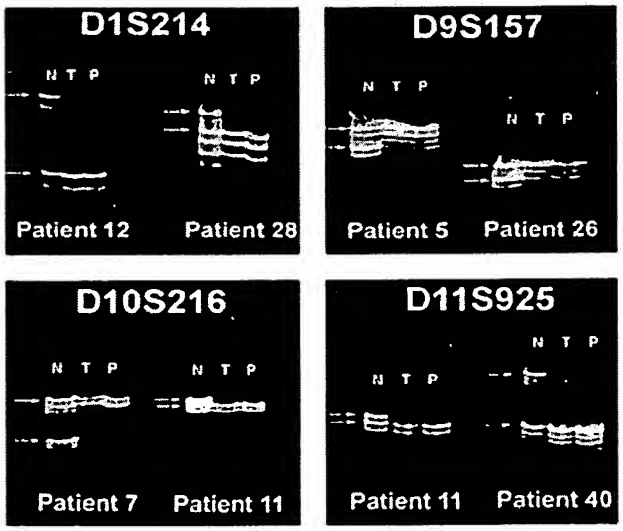

Fig. 2. Allelic losses in tumor and plasma at individual microsatellite loci. Analysis for patients-paired specimens: *N*, lymphocytes; *T*, melanoma; *P*, plasma. *Arrows*, the position of the deleted alleles or LOH. Microsatellite markers are indicated below.

disease: 5 of 19 (26%) patients with stages I and II had LOH, whereas 33 of 57 (58%) patients with stages III and IV had LOH in at least one locus. One interesting finding was that in a stage I patient's plasma and primary tumor, LOH detected at loci *D3S1293* and *D10S212* showed a different pattern of allele loss in the tumor and plasma (Fig. 3). The longer allele was deleted in both plasma and tumor for *D3S1293*, whereas for *D10S212*, LOH was at the longer allele in the plasma and at the shorter allele in the tumor. The discrepancy may be due to the heterogeneity of the primary tumor or the presence of subclinical metastasis. Clinical follow-up for disease recurrence may help in determining the cause of these events.

Statistical analyses were performed to determine whether the frequency of LOH in plasma correlated to clinical stage and known melanoma prognostic factors. Assessment of clinical correlation between stage and individual microsatellite markers showing LOH in the patients' plasma was performed. Overall, there was a significant correlation between the number of LOH microsatellite markers detected within a patient's plasma and AJCC stage in the 76 patients ($P = 0.02$). Only at loci *D3S1293* was there a significant correlation ($P = 0.02$) between LOH detection and clinical progression of disease. The next closest correlation with an individual marker was at D1S228 ($P = 0.065$). In the correlation between the progression of different clinical stages of disease and microsatellite marker combinations showing LOH, the combinations of D9S157 and D3S1293 ($P = 0.01$), D9S157 and D1S228 ($P = 0.05$), and D11S925 and D3S1293 ($P = 0.01$) were the most significant. These correlations were independent of known prognostic factors for melanoma. The microsatellite marker with LOH combination of D1S228 and D3S1293 showed a trend toward significance ($P = 0.07$).

We also compared LOH in plasma DNA with other clinicopathological parameters and clinical status of disease (no evidence of disease or alive with disease) at the time of blood collection. There was no significant correlation between the frequency of LOH in

Table 2 *Microsatellite analysis and clinical status of melanoma patients*

Patient number	Tumor LOH	Plasma LOH	AJCC stage at blood draw	Clinical status at blood draw	Total follow-up (month)	Disease progression[a]
1	+[b]	−[c]	4	AWD[d]	3.4	+
2	+	+	4	NED	10	+
3	+	−	4	AWD	14.3	+
4	+	+	4	NED	14.7	−
5	+	+	4	NED	14.9	−
6	+	−	4	AWD	14.5	+
7	+	+	4	NED	8.5	+
8	+	−	4	NED	11.4	+
9	+	−	3	NED	14.3	+
10	−	+	3	NED	13.8	−
11	−	+	3	NED	14.7	−
12	−	+	3	NED	12.7	−
13	−	+	4	NED	14.8	−
14	−	+	3	NED	12.9	+
15	+	−	3	AWD	14.2	+
16	+	−	3	NED	7.5	+
17	+	−	3	NED	14.5	+
18	+	+	3	NED	14.3	+
19	+	−	3	NED	14.8	+
20	+	−	4	NED	10.6	+
21	+	+	4	AWD	1.9	+
22	−	−	4	AWD	9.7	−
23	+	−	4	AWD	14.8	−
24	−	−	4	AWD	9.6	−
25	−	−	4	NED	13.6	+
26	+	+	3	NED	13.6	+
27	+	+	3	NED	14.1	−
28	+	+	3	NED	12.0	−
29	+	+	4	AWD	3.4	+
30	+	−	3	NED	13.9	−
31	+	−	4	NED	14.4	−
32	+	+	4	AWD	14.5	+
33	+	+	2	NED	14.9	+
34	+	+	4	NED	10.7	+
35	+	+	4	AWD	14.2	+
36	+	+	4	AWD	15.8	+
37	−	+	4	AWD	6.8	+
38	−	−	3	NED	14.8	−
39	+	+	4	NED	4.5	+
40	−	+	4	AWD	3.7	+

[a] Disease progression refers to expiration, recurrence, increase in number of metastases or tumor size; yes (+) and no (−).
[b] LOH was detected at least at one locus.
[c] LOH was not detected at any loci.
[d] AWD, alive with disease; NED, no evidence of disease.

Table 3 *Correlation of LOH in patients' plasma to AJCC stage*

AJCC stage	LOH +[a]	LOH−	Total number of patients
I	2 (29%)	5 (71%)	7
II	3 (25%)	9 (75%)	12
III	16 (53%)	14 (47%)	30
IV	17 (63%)	10 (37%)	27

[a] At least one locus detected has LOH.

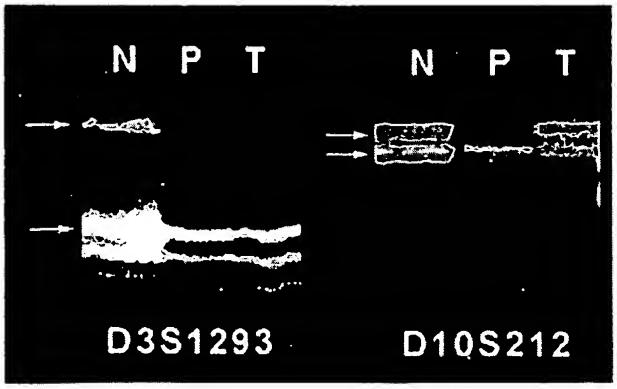

Fig. 3. Different allelic pattern shown in a matched AJCC stage I patient's primary tumor lesion and plasma. Analysis of individual patient's DNA: *N*, lymphocytes; *P*, plasma; *T*, microdissected primary melanoma lesion. *Arrows*, the position of the allele bands.

plasma or tumor, and standard prognostic factors such as Breslow's thickness or Clark's level. Patients were also assessed for correlation of LOH presence with recurrence or progression of disease (Table 2), and because the mean follow-up of patients is only about 1 year, the results cannot be appropriately evaluated.

DISCUSSION

The development of molecular markers is needed to improve diagnosis and prognosis of disease and to assess tumor progression in melanoma patients. Better profiles of tumor genetic changes are needed to determine mechanics associated with disease progression. We and others have examined molecular markers such as melanoma-associated antigen mRNA markers in RT-PCR assays to detect melanoma cells in blood, lymph nodes, and various other organs (38, 39). These molecular markers have been shown to be very useful in detecting metastatic melanoma cells and disease progression. Although these RT-PCR-based markers have a high level of sensitivity, there are still some limitations, such as potential false positives and the expression of mRNA markers by normal cells. Other limitations in conducting RT-PCR analysis for large clinical studies are the high potential of RNA contamination and integrity of mRNA available. The mRNA expression level of a particular tumor can vary among tumor cells, which also reduces the sensitivity of the assay. However, from these studies, we have determined that multiple molecular markers are needed in assessment of melanoma progression. This approach takes into consideration the heterogeneity of tumors and the dynamics of tumor genetic changes occurring during tumor progression.

Analysis of DNA markers for somatic mutations of tumor suppressor genes and oncogenes and chromosome alterations are more specific to tumors and require less logistical stringency than mRNA analysis. The frequency of known tumor suppressor genes or oncogenes with mutations in melanoma is <25% individually, which makes them unsuitable as molecular markers for detection overall. DNA mutations of the *K-Ras* gene, previously reported (Y. F.) to be present in the plasma of patients with colorectal and pancreas cancer, has been correlated to clinical status (22). However, this molecular marker occurs quite frequently in these cancers, but not in melanoma. DNA cancer-cell specific markers do not have many of the problems associated with mRNA markers. In melanoma, loss of loci on specific chromosome arms is one of the most common genetic events occurring in these tumors. The correlation of LOH markers to clinical progression or prognosis has been shown in other cancers, but has not been well described for melanoma. The assessment of a combination of microsatellite markers with high frequency of LOH as genetic markers in blood may be very useful for monitoring melanoma progression at different stages of disease. Blood is logistically practical for the monitoring of multiple patients sampled at different time points during tumor progression. Traditional approaches using embedded tissue for analysis of LOH are not always practical and, as a result, there are major gaps of information missing on events during tumor progression.

The panel of 10 microsatellite markers used in the study showed LOH with at least one marker in 85% of the melanoma tumors assessed. Twenty-three of 34 (68%) patients who showed LOH for at least one marker in tumor also showed LOH in plasma. These analyses of matched patients' plasma and tumor were highly statistically concordant for all microsatellite markers studied. The assay is very specific in that none of the normal samples tested showed LOH at any loci. The frequency and combinations of LOH at specific microsatellite loci can vary considerably among tumors with different histological origin. These results demonstrate that tumor-specific DNA is released frequently into the blood circulation and remains stable in melanoma patients. In contrast to mRNA, specific tumor-derived DNA is more stable in blood and is more resistant to rapid degradation. Previous studies revealed that the serum of cancer patients contains approximately two to four times the amount of free DNA as that of normal donors (23, 40). Future studies will help determine the half-life of these microsatellite markers and whether specific markers stay longer in blood circulation (*i.e.*, resistant to degradation). The remarkable finding is that the LOH of the individual microsatellite markers can be detected easily in the plasma. The mechanism of how DNA (LOH marker) is released into the blood circulation is unknown. This can be related to cell death and necrosis in tumors and/or destruction of tumor cells circulating in blood. A significant correlation was observed within individual patients who showed LOH in plasma and paired tumor microsatellite markers. This demonstrated the relative detection sensitivity from a single bleed. Studies on several bleed samples over several months from the same patient with LOH in plasma have shown consistency in presence of the marker.

The frequency and the number of microsatellite markers with LOH in plasma significantly increased in more advanced clinical stages of disease. These findings support previous work on melanoma and other cancers that show that as tumors progress, the number of genetic changes accumulates. Only at loci *D3S1293* was LOH significantly correlated to disease progression. Located at this site are the tumor suppressor gene *VHL* (3p25-p26) and other genetic abnormalities that are frequently found in renal cell carcinomas (41). Interestingly, two of the most frequently and well studied microsatellite loci in melanoma, *9p21* and *6q*, did not correlate with disease progression individually. This may be due to the limited number of patients analyzed in the study. LOH of these two markers has been detected at early stages of melanoma development therefore, the loci alone may not be significantly correlated with later stages of tumor progression. Combinations of specific LOH markers may be necessary for tumor progression to be successful. One of the major problems in interpretations of these analyses is that tumor progression clinicopathology at later stages is highly variable. A recent study on LOH of microsatellite marker at 6q of melanomas showed a correlation with poorer clinical outcome (16). The combinations of genetic changes are more likely to be correlative to disease progression. In our study, LOH at 9p combined with LOH at 3p or 1p showed significant correlation. Assessment of LOH at 3p alone or in combination showed better correlation with disease progression than any other marker.

This study illustrates the clinical se of microsatellite analysis in detecting tumor DNA in plasma of melanoma patients. The analysis of

LOH in plasma provides a logistically practical assay to monitor genetic changes during melanoma progression. The study demonstrates that at early clinical stages, release of DNA (LOH marker) is limited. Plasma LOH analysis may be more suitable to monitor stage II to stage IV progression before and during therapy as well as during posttreatment follow-up. The markers may be also useful to detect subclinical disease recurrence in disease-free patients. Tumor progression is dynamic, and the genetic changes that concurrently occur are also ongoing. The most significant advantage of this approach in assessing plasma compared with direct analysis of tumor biopsies is the ability to monitor disease progression and genetic changes without assessing the tumor. This is particularly important during early phases of distant disease spread in which subclinical disease is undetectable by conventional imaging techniques. Additional retrospective and prospective analyses are being carried out to determine the prognostic significance of the DNA microsatellite markers during treatment and as overall predictors of disease outcome.

ACKNOWLEDGMENTS

We thank the clinical staff of John Wayne Cancer Institute clinic for blood procurement.

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Exhibit B

(1) changes in the cell membrane impeding drug transport (e.g. of methotrexate), (2) DNA repair of drug-induced lesions (e.g. caused by alkylating agents), (3) utilisation of alternative metabolic pathways (e.g. 5-fluorouracil), (4) increased production of a target enzyme (e.g. dihydrofolate reductase binding to methotrexate), or (5) modification of the target enzyme, enabling it to recognise the difference between true and false metabolites. Irreversible binding between enzyme and cytotoxic drug (e.g. 6-mercaptopurine) is thus avoided.

The multiple drug resistance gene (*MDR1*) encodes P-glycoprotein. The latter is a membrane-associated efflux pump which serves to protect normal cells in the testis and kidney from drug-induced damage. Cancer cells may overexpress *MDR1* so conferring resistance to a variety of chemotherapeutic agents. In addition *p53*, the 'guardian' of the genome and an important mediator of apoptosis (programmed cell death) may be mutated and give rise to chemoresistance in a number of solid tumours.

The reasons for drug resistance are not fully understood. It is common to find that a tumour responds to a particular drug or combination of drugs for a period of time and then ceases to do so. It is thought that within many tumour populations there are genetically determined drug-resistant cells. When the chemosensitive cells have been killed, the resistant population may proliferate.

Drug resistance to repeated exposure to a single may result from changes in drug metabolism, inactivation of a drug or of cofactors.

Resistance to cisplatin is considered to be mediated by increased DNA repair. The cytotoxic action of cisplatin is known to be due to lethal intrastrand DNA crosslinks. It is known that DNA synthesis increases in ovarian cancer cells resistant to cisplatin. Inhibitors of DNA polymerase, which is involved in DNA repair, are under investigation to try to overcome resistance to cisplatin.

Some drugs which show excellent cell kill in vitro fail to do so in vivo. Sometimes this is due to a sanctuary site of tumour in the CNS where the drug does not cross the blood–brain barrier. Occasionally the tumour outstrips its vascular supply so that inadequate concentrations of drug reach the tumour. There is also some evidence that some tumours exhibit drug resistance that is in part due to host factors which modify the pharmacokinetics of the anticancer agent in vivo.

Chemotherapy, as radiotherapy, is most effective in killing proliferating cells. While the growth fraction is high in many chemosensitive tumours, such as the lymphomas and testicular teratomas, it is relatively low in many common tumours, e.g. colorectal cancer.

In parts of the tumour the blood supply tends to be poor. As a result concentrations of drug reaching the tumour may be inadequate. In addition, as a result of the hypoxia induced by a poor blood supply, the growth fraction is reduced.

agent will usually result in cross-resistance to other compounds of the same class of drugs. This is probably due to common transport mechanisms and pathways of metabolism and intracellular cytotoxic targets. However, cancer cells which have become resistant to one class of drugs may retain sensitivity to another class of drugs.

Some drugs may have a variety of mechanisms of drug resistance. The anthracyclines (e.g. doxorubicin) is thought to derive some of its cytotoxicity from the formation of free radical intermediates. One of these free radicals, the superoxide anion, gives rise to the highly reactive hydroxyl radical, which damages DNA directly. One of the mechanisms of resistance to anthracyclines may be impaired superoxide anion levels in the tumour owing to poor tumour vascularity. Alternatively, enhanced repair of peroxidative damage to DNA may account for some of the resistance to anthracylines.

Reduced accumulation of a drug may be due to reduced influx (e.g. of methotrexate), impaired membrane transport (resistance to nitrogen mustard), increased drug efflux (multidrug resistance). Resistance

A number of approaches are being pursued to try to overcome drug resistance. These include giving very intensive initial chemotherapy to try to eradicate the whole tumour cell population. Attempts are being made to develop anti-P glycoprotein antibodies either bound to toxins or to complement which can target P glycoprotein-positive cells.

Adjuvant and neoadjuvant chemotherapy

Even when the primary tumour appears to be localised, clinically undetected metastases (micrometastases) may already have seeded to distant sites. The classical example is breast cancer where the lungs, liver and bone may be infiltrated by metastases. This is particularly so if the axillary nodes are involved at the time of primary surgery. Chemotherapy aimed at eradicating these micrometastases is called *adjuvant*. Other tumours in which the elimination of micrometastatic spread by adjuvant chemotherapy may be possible include colon cancer, ovarian cancer, osteogenic sarcoma and Ewing's sarcoma of bone.